Multiple interactions between SRm160 and SR family proteins in enhancer-dependent splicing and development of C. elegans

Multiple interactions between SRm160 and SR family proteins in enhancer-dependent splicing and development of *C. elegans*

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Background: SR family and SR-related proteins assemble on exonic splicing enhancer (ESE) sequences to promote both constitutive and regulated splicing. The SRm160 splicing coactivator, an SR-related nuclear matrix protein of 160 kDa, is important for the splicing of specific constitutive and ESE-dependent pre-mRNAs.

Results: In the present study, we show that SRm160 is required to promote pre-mRNA splicing mediated by a large population of functional ESE sequences within a randomized 18 nucleotide sequence. This suggests that it functions as a general coactivator by interacting with different SR family/SR-related proteins bound to different ESE sequences. Consistent with this, several SR family and SR-related proteins coimmunoprecipitated specifically with SRm160 in the presence of low salt. We used RNA interference (RNAi) in *Caenorhabditis elegans* to determine whether interactions between CeSRm160 and different CeSR family proteins are important in a whole-organism context. Previously we showed that RNAi of CeSRm160 and individual CeSR family genes other than CeSF2/ASF results in no obvious phenotype, which is indicative of gene redundancy. In the present study, we demonstrate that RNAi of CeSRm160 in combination with any CeSR family gene results in the production of unfertilized oocytes by the injected mother.

Conclusions: The observation that simultaneous suppression of CeSRm160 and individual CeSR family proteins results in a distinct phenotype is indicative of critical functional interactions between these factors. Our results provide biochemical and genetic evidence indicating that interactions between SRm160 and multiple SR family proteins are important for both optimal splicing activity and for proper development.

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Background

Pre-mRNA splicing takes place within the spliceosome, a large molecular complex composed of four small nuclear ribonucleoproteins (U1, U2, U4/U6, and U5 snRNPs) and numerous non-snRNP factors (for a review, see [1]). The most extensively characterized non-snRNP splicing factors are SR family proteins, a group of structurally and functionally related proteins which have a dual role in both constitutive and alternative splicing [2, 3].

The SR family comprises a group of highly conserved proteins in metazoans, with a modular structure consisting of one or two RNA recognition motifs (RRMs) and a C-terminal domain rich in alternating serine and arginine residues (RS domain). SR family proteins function early in spliceosome formation and are involved in multiple steps of the splicing reaction. For example, they facilitate the recruitment of the U1 snRNP to the 5′ splice site [4]. SR family proteins also bridge the 5′ and 3′ splice sites via RS domain-mediated interactions involving the U1 snRNP-associated protein U1-70K at the 5′ splice site and the 35 kDa subunit of the heterodimeric splicing factor U2AF-65/35, which binds at the 3′ splice site [5]. The SR family proteins also participate at later stages of the splicing reaction, when they facilitate the recruitment of the U4/U6.U5 tri-snRNP complex [6]. Another class of RS domain-containing proteins, termed SR-related proteins or SR protein-related-polypeptides (SRrps), are also involved in splicing. This group comprises RS domain-containing proteins that are structurally distinct from the SR family and may or may not contain RRMs. Examples are the U1-70K protein and both 35 and 65 kDa subunits of U2AF as well as several alternative splicing regulators, including Tra, Tra2, and SWAP (for a review, see [7, 8]).

In addition to their many roles in constitutive splicing, SR family proteins are also important regulators of alternative splicing and in many cases function to antagonize the activity of hnRNP A/B proteins in splice site selection. For example, elevated concentrations of SR family proteins result in the selection of intron-proximal 5′ splice sites, whereas an excess of hnRNP A/B proteins promotes the

selection of intron-distal 5′ splice sites [9–13]. Thus, the relative levels and activities of these two families of antagonistic factors plays a critical and widespread role in the regulation of splice site selection [14] (reviewed in [15]). SR family and SR-related proteins function in the recognition of exonic splicing enhancers (ESEs) which activate use of adjacent, suboptimal 3′ or 5′ splice sites [16]. In some cases, it has been found that binding of SR family proteins to ESEs functions to overcome the negative activity of exonic splicing silencer (ESS) elements that bind to hnRNP proteins [17, 18].

Mammalian SRm160/300 is a protein complex, consisting of two SR-related nuclear matrix proteins of 160 and 300 kDa, that functions as a coactivator of splicing [19, 20]. The SRm160/300 proteins both contain RS domains but lack recognizable RRMs and do not normally bind to pre-mRNA in the absence of other splicing factors. The association of SRm160/300 with pre-mRNA requires U1 snRNP, SR family proteins, and is stabilized by U2 snRNP [19, 20]. Depletion of SRm160/300 but not of SRm300 alone prevents splicing of a subset of constitutive pre-mRNAs as well as ESE-dependent splicing of a *dsx* pre-mRNA. Moreover, addition of purified recombinant SRm160 to SRm160/300-depleted reactions can restore splicing activity, indicating that SRm160 is the more important component of this complex for splicing [21]. These studies led to the proposal that SRm160 functions as a splicing "coactivator" by mediating interactions between one or more SR family and/or SR-related protein "activators" bound to ESEs and basal splicing factors, including U1 and U2 snRNP components [16].

In this study, we provide biochemical and genetic evidence for the existence of multiple functional interactions between SRm160 and different SR family proteins. A large population of functional ESEs sequences within a randomized 18 nucleotide sequence, inserted within an ESE-dependent *dsx* pre-mRNA, required SRm160 for splicing activity. Consistent with a more general coactivator role for SRm160, several SR family and SR-related proteins specifically coimmunoprecipitated with SRm160 in the presence of low salt, demonstrating extensive protein-protein interactions between these splicing components. RNA interference (RNAi) experiments in the nematode *C. elegans* provided evidence for critical interactions between SRm160 and SR family proteins in the development of this organism. Simultaneous RNAi of SRm160 and any one of the SR family proteins resulted in a specific defect, leading to the production of unfertilized oocytes by the injected animal. These results support the existence of conserved interactions between SRm160 and multiple SR family proteins and provide evidence that these interactions are important for splicing as well as for proper development.

Results

Function of SRm160 in ESE-mediated splicing

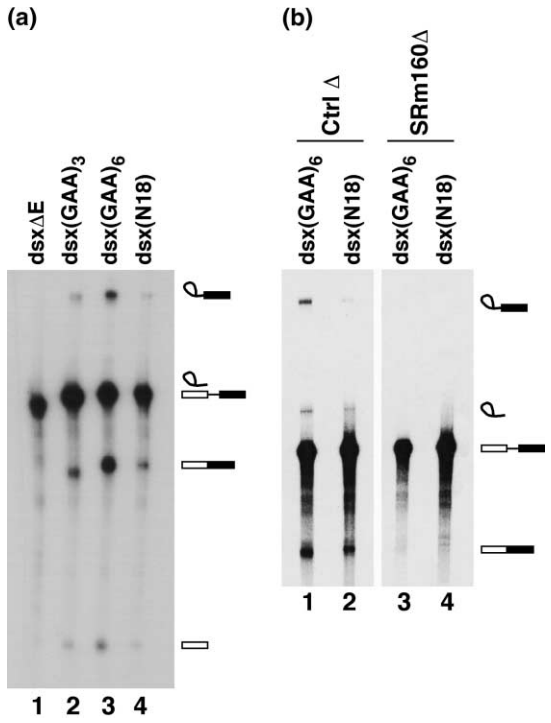
In previous studies, it was demonstrated that the SRm160 subunit of the SRm160/300 splicing coactivator is important for a typical mammalian ESE, consisting of six GAA repeats, to promote the splicing of a pre-mRNA derived from exons 3 and 4 of the *Drosophila doublesex* gene [*dsx*$(GAA)_6$]. This substrate contains a suboptimal 3′ splice site and requires an ESE for efficient splicing. It was found that the 6×GAA ESE promoted the efficient association of SRm160 with the *dsx* substrate, consistent with an important role for this factor in mediating ESE-dependent splicing [20–22]. Immunodepletion of SRm160/300 but not of SRm300 alone prevented the 6×GAA ESE-dependent splicing of this substrate but not the low level of splicing detected in the absence of the 6×GAA ESE. Moreover, addition of recombinant SRm160 could restore splicing activity to SRm160/300-depleted reactions [21].

These previous studies did not determine whether SRm160 is important for the promotion of splicing by other types of ESE sequences. For example, SRm160 could function as a more general coactivator that promotes the activity of many different ESE sequences or, alternatively, as a specific coactivator that only functions in conjunction with a specific subset of ESE sequences. In order to distinguish between these possibilities, we prepared a *dsx* pre-mRNA that contains a randomized 18 nucleotide sequence in place of the 6×GAA ESE sequence [*dsx*(N18)]. Sequencing of the *dsx*(N18) template confirmed that each position within the randomized region was represented equally by all four bases. The splicing efficiency of the *dsx*(N18) substrate was first compared alongside the *dsx*$(GAA)_6$ pre-mRNA, a *dsx* pre-mRNA containing an ESE of intermediate activity consisting of 3×GAA repeats [*dsx*$(GAA)_3$], and a *dsx* substrate lacking an ESE (*dsx*ΔE) (Figure 1a). Consistent with previous reports [21, 22], the 6×GAA ESE promoted relatively efficient splicing of the *dsx* pre-mRNA, resulting in ~37.1% of the pre-mRNA being converted to mRNA product (Figure 1a, lane 3). In contrast, the *dsx*ΔE pre-mRNA was spliced poorly (~0.6%) (lane 1), and an intermediate level of splicing was observed for the *dsx*$(GAA)_3$ pre-mRNA (~14.3%) (lane 2). Consistent with previous estimates of the percentage of functional ESE sequences within a randomized 18 or 20-mer (ranging from 15% to 20% [23, 24], ~16.5% of the *dsx*(N18) pre-mRNA was spliced, indicating that a large population of the sequences within the randomized N18-mer function to promote splicing of the *dsx* pre-mRNA (Figure 1a, lane 4).

A broad spectrum of ESE sequences require SRm160/300 for function

The level of *dsx*(N18) pre-mRNA splicing was next compared in nuclear extracts specifically immunodepleted of

Figure 1



SRm160/300 is important for the majority of functional ESEs within a random 18-mer to promote splicing. **(a)** In vitro splicing of different radiolabeled pre-mRNA substrates derived from the *doublesex* gene of *Drosophila*. The pre-mRNAs either lacked an ESE (*dsx*ΔE, lane 1), contained an ESE in exon 4, consisting of three GAA repeats [*dsx*$(GAA)_3$, lane 2] or six GAA repeats [*dsx*$(GAA)_6$, lane 3], or a randomized 18-mer [*dsx*(N18), lane 4] in place of an ESE. The radiolabeled *dsx* pre-mRNAs were incubated in splicing reactions for 60 min. RNA recovered from the reactions was analyzed on a 15% denaturing polyacrylamide-urea gel. The RNA intermediates and products of the splicing reaction are indicated. **(b)** The majority of functional ESEs within a random 18-mer require SRm160 for splicing. Radiolabeled *dsx*$(GAA)_6$ (lanes 1 and 3) and *dsx*(N18) (lanes 2 and 4) substrates were incubated in splicing reactions containing nuclear extract that was specifically depleted of SRm160/300 (lanes 3 and 4) or mock depleted with preimmune serum (lanes 1 and 2). RNA recovered from the splicing reactions was analyzed as in (a).

SRm160/300, or "mock"-depleted with a control antibody (Figure 1b). The SRm160/300-depleted nuclear extract used in this experiment was shown by immunoblotting to be specifically immunodepleted of these factors but not of other SR-related or SR family proteins and could be reconstituted for splicing activity by the addition of highly purified, recombinant SRm160 ([19, 21]; data not shown).

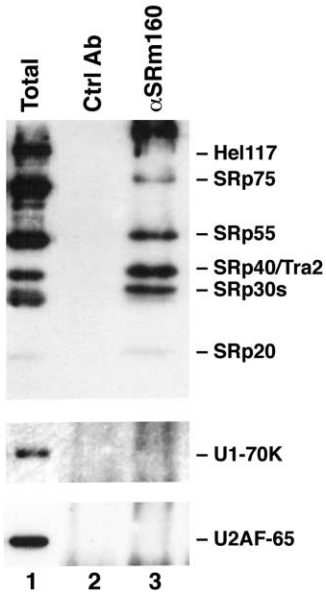
Significantly, splicing of the *dsx*(N18) substrate was strongly inhibited in the absence of SRm160/300 (Figure 1b, lane 4). Compared to the mock depleted reaction, in which ~15.7% of the *dsx*(N18) pre-mRNA was converted to mRNA product, only ~0.8% of this substrate was converted to mRNA product in the SRm160/300-depleted reaction (compare lanes 2 and 4). Similarly, depletion of SRm160/300 resulted in a reduction from 28.2% to 0.4% splicing of the *dsx*$(GAA)_6$ pre-mRNA (compare lanes 1 and 3). This indicates that ~95% of functional ESE sequences within the *dsx*(N18) substrate require SRm160 for function. A portion of the low level of splicing that still occurs in the SRm160/300-depleted reactions may be due to the low level of SRm160 remaining after immunodepletion and/or the low level of splicing that still occurs on this substrate in the absence of an ESE (e.g., Figure 1a, lane 1, and data not shown). Although we have not determined the identity of individual sequences within the *dsx*(N18) pre-mRNA which require SRm160, the results indicate that a large population of functional ESE sequences within a random 18 nucleotide sequence require SRm160 to promote splicing of the *dsx* pre-mRNA.

In conjunction with previous studies on the mechanism of ESE-dependent splicing [23, 24], the results in the present study suggest that SRm160 could functions as a more general coactivator by promoting splicing of a large population of different ESE sequences. If this model is correct, it would be expected that SRm160 associates with the majority of pre-mRNAs in the *dsx*(N18) population that contain a functional ESE sequence. This was indeed found to be the case, since the relative levels of splicing complexes immunoprecipitated with the anti-SRm160 monoclonal antibody (mAb-B1C8) on the *dsx*(N18) versus the *dsx*$(GAA)_6$ substrate correlated well with the difference in the splicing activity between these two substrates (data not shown).

SRm160 associates with multiple SR family and SR-related proteins

In order for SRm160 to function as general coactivator of ESE-dependent splicing, it would also be expected that it associates with many different SR family and/or SR-related proteins that promote splicing by binding to different ESE sequences. In previous studies, it was found that antibodies to SRm160 preferentially coimmunoprecipitated from HeLa nuclear extract 75 kDa and 40 kDa proteins that react with the anti-SR protein monoclonal antibody mAb104 [19]. The 75 kDa protein probably corresponded to the SR family protein of this size, SRp75, whereas a significant fraction of the 40 kDa protein was subsequently found to correspond to hTra2-β [20]. However, the previous immunoprecipitation experiments were performed under relatively high salt conditions (~0.5 M) that may have resulted in the dissociation of additional SR family and/or SR-related proteins that interact with SRm160 under splicing conditions (<0.1 M salt). To address this possibility, we used the anti-SR protein monoclonal antibody (mAb104) to detect SR proteins that coimmunoprecipitate with SRm160 from HeLa nuclear extracts in the presence of 0.1 M salt [25]. Similar to the previous results, "SRp75" and hTra2-β were detected in immuno-

Figure 2



SRm160 associates with several SR proteins recognized by the monoclonal antibody mAb104. Immunoprecipitates were collected with a monoclonal antibody specific for SRm160 (B1C8, [26]) (lane 3) and a control antibody (murine IgM) (lane 2). Immunoprecipitated proteins were separated on a 12% SDS polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with mAb104 [25] or antisera specific for U1-70K and U2AF-65 [48]. Total nuclear extract separated in lane 1 represents ~20% of the amount of nuclear extract used in each immunoprecipitation in lanes 2 and 3. Bands corresponding to U1-70K, U2AF-65, and the defined SR family and SR-related proteins recognized by mAb104 are indicated. The pronounced band migrating above Hel117 corresponds to coimmunoprecipitated SRm160.

precipitates prepared with the murine monoclonal antibody specific for SRm160 (mAb-B1C8) [26] ("αSRm160"; Figure 2, lane 3). In addition, a low level of a protein migrating at ~130 kDa was coimmunoprecipitated, as well as proteins of 55, 30, and 20 kDa. A 130 kDa antigen detected by mAb104 has recently been identified as the SR-related DEAD-box protein Hel117 [27, 28], and the 55, 30, and 20 kDa antigens probably correspond to one or more of the defined SR family proteins of these sizes, including SRp55, SC35, 9G8, SRp30c, SF2/ASF, and SRp20. Immunoblotting of mAb-B1C8 immunoprecipitate with an antibody specific for Hel117 [28] confirmed the identity of the mAb104 130 kDa antigen as this protein (data not shown). Immunoprecipitation of the different SR family and SR-related proteins with mAb-SRm160 was highly specific, since two other splicing factors which contain RS domains, U1-70K and U2AF-65, were not coimmunoprecipitated (Figure 2). Moreover, none of the SR family or SR-related proteins were immunoprecipitated to an appreciable extent with a control serum (murine IgM) (Figure 2, lane 2). The coimmunoprecipitation of these proteins with mAb-B1C8 antibody was not reduced by extensive pretreatment of the nuclear extract with ribonuclease, indicating that all of the SR family and SR-related antigens detected with mAb104 are associated with SRm160 through protein-protein interactions (data not shown). These results are consistent with a general coactivator role for SRm160, in which it promotes splicing activity by associating with different SR family and/or SR-related proteins bound to distinct ESE sequences.

RNAi of SRm160 in combination with individual SR proteins causes a progressive maternal phenotype resulting in the production of deficient oocytes

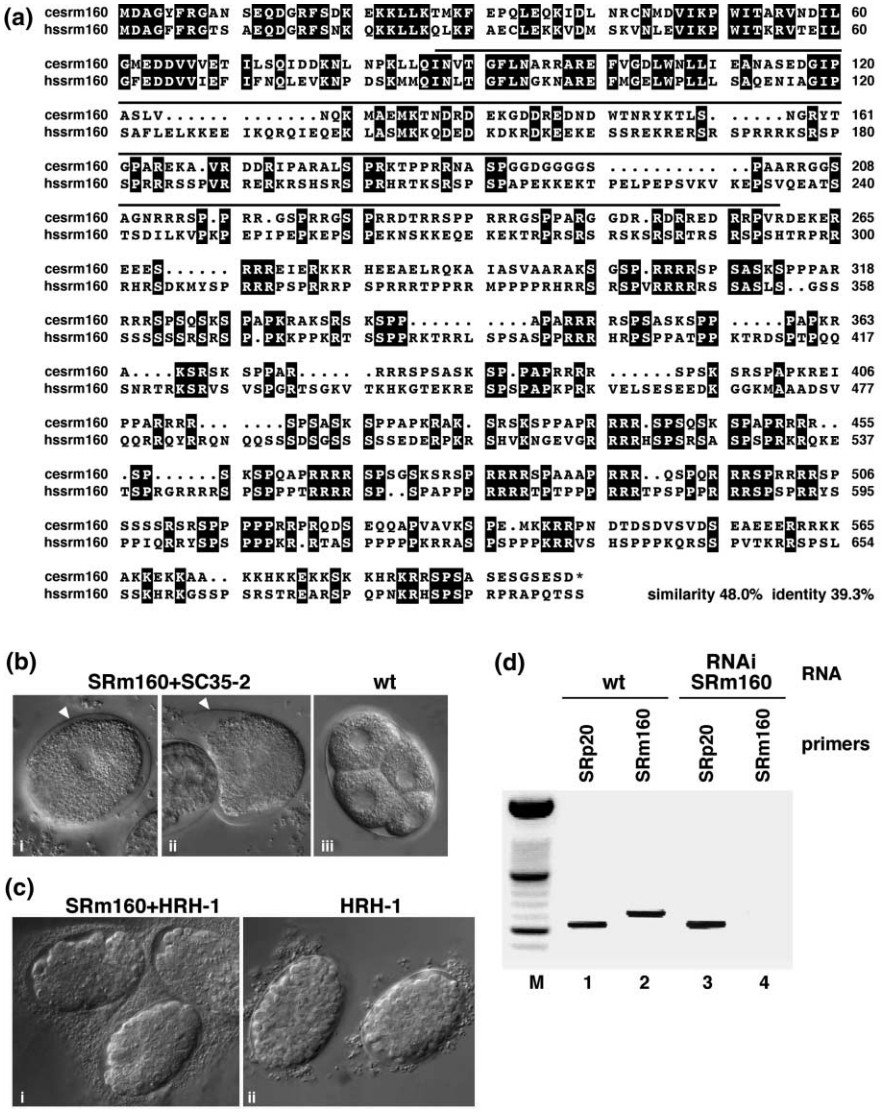
We next sought to determine whether interactions between SRm160 and different SR proteins are important in vivo in a whole-organism context. To this end, we have used RNA interference (RNAi) in the nematode *C. elegans* to ask whether selectively interfering with the expression of genes encoding the homologs of SRm160 and different SR family proteins affects the development of this organism.

SR family proteins are highly conserved throughout metazoans, and individual members of the family display higher homology across species than among family members within the same species. From database searching, we previously identified seven candidate homologs of human SR family genes as well as a candidate homolog of SRm160 in the *C. elegans* genome [29] (these are designated with the prefix "Ce" below). The CeSRm160 ORF (*rsr-1*) displays high homology to the human SRm160 protein sequence (48.0% similarity and 39.3% identity; Figure 3a), despite the fact that it corresponds to a shorter protein (601 versus 820 amino acids). This high degree of conservation is evident throughout the different motifs of the protein, including the N-terminal PWI motif, a domain of unknown function that is shared with other splicing-related proteins [30], and several repeat motifs that are rich in arginine, serine, and proline residues. For example, like human SRm160, CeSRm160 contains an RS domain consisting of numerous SR/RS dipeptides, several of which are clustered (Figure 3a, and Figure S1b available as Supplementary material with this article online).

We previously showed that, whereas RNAi with CeSF2/ASF (*rsp-3*) caused late embryonic lethality, RNAi of other CeSR family proteins or of CeSRm160 resulted in no obvious phenotype. This suggested that these CeSR family proteins and CeSRm160 have redundant functions [29].

RNAi can be used to simultaneously inactivate more than one gene product; thus, combinatorial RNAi can be used as a powerful tool to study "genetic" interactions and to test whether different gene products function in common pathways [31, 32]. In the case of the SR family, it was shown that RNAi of certain combinations of CeSR genes

Figure 3



(a) New analysis revealed that the CeSRm160 protein is longer than the original AceDB prediction (identifier F28D9.1) and corresponds to the translation of Genie gene prediction g-I-2048 (see Figure S1). CeSRm160 predicted protein and human SRm160 protein sequences were compared using the GAP program (GCG10 software), and the alignment was generated using PRETTYBOX (GCG10 software). Identical residues are highlighted in black, and the dsRNA fragment used for RNAi corresponds to the solid line above the sequence. **(b)** RNA interference with a combination on SRm160 and SC35-2 genes. Panels i and ii show deficient oocytes laid by the injected worms, which lack an egg shell (arrowhead). Panel iii shows a wt embryo at the four-cell stage for comparison. **(c)** RNAi with a combination of CeSRm160 and CeHRH-1 genes (panel i) gave rise to an identical early embryonic lethal phenotype as the one obtained by RNAi with HRH-1 alone (panel ii). Each embryo is ~50 μm in length. **(d)** The effectiveness of RNAi was determined by examining the level of the residual transcripts following dsRNA injections by RT-PCR with specific primers, as previously described [29]. CeSRm160 mRNA is specifically depleted in RNAi-treated animals (lane 4) compared to wt animals (lane 2), whereas the level of a control mRNA, corresponding to CeSRp20, is unaffected (lanes 1 and 3). The figure shows a negative of an ethidium bromide-stained agarose gel. M, 100 bp ladder DNA size marker.

resulted in specific developmental defects, indicating common functions for those proteins [29, 33]. In the present study, we applied a combinatorial RNAi approach to investigate genetic interactions between CeSRm160 and individual CeSR family proteins.

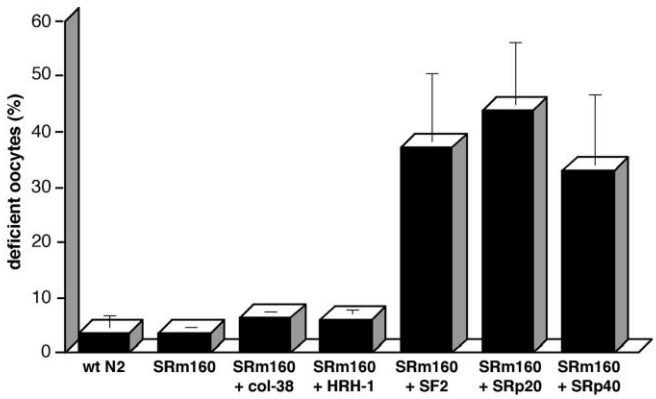
As previously observed, RNA interference of CeSRm160 showed no obvious phenotype. In contrast, RNAi of CeSRm160 (*rsr-1*) in combination with CeSC35-2 *(rsp-5)*, one of two *C. elegans* genes displaying high homology to mammalian SC35, caused a specific defect, resulting in the production of deficient oocytes by the injected animal (Figure 3b, panels i and ii). The injected mothers laid a large number of oocytes, which did not develop further. RT-PCR analysis of animals individually suppressed for the CeSRm160 gene showed that the targeted mRNA was efficiently depleted in the F1 progeny, whereas a control RNA (CeSRp20) was present at levels comparable to those detected in wild-type animals (Figure 3d). This indicated that the RNAi treatment was specific and highly efficient.

A similar maternal, nonzygotic phenotype was observed with RNAi of CeSRm160 in combination with any one of the CeSR family genes, including CeSRp20, CeSRp40, and CeSF2/ASF (Figure 4a,b and data not shown). This deficient-ooctyte phenotype is progressive, since it becomes more evident with longer incubation times (data not shown). Typically, all injected animals displayed the phenotype on the third day after the injection. In each case, the penetrance of this phenotype was similar, resulting in ~40% of deficient oocytes within the scored progeny (Figure 4 and data not shown). When simultaneously interfering with the expression of CeSRm160

Figure 4

RNA interference with CeSRm160 and individual CeSR family proteins. **(a)** Graph indicating the level of production of deficient oocytes (*y* axis) after RNA interference, with genes indicated on the *x* axis. RNAi with SRm160 in combination with any SR family gene leads to a dramatic increase in the production of deficient oocytes compared to either wild-type worms, RNAi with SRm160 alone, or RNAi with SRm160 in combination with *col-38* or HRH-1. (**b**) Table describing the RNA interference phenotypes.

(a)



(b)

Genes	RNAi phenotype	Production of deficient oocytes (%)
wt N2	–	3.5 ± 3.3
SRm160	no phenotype	3.7 ± 0.4
SRm160 + col-38	no phenotype	6.3 ± 1.1
HRH-1	early embryonic lethality	nd
SRm160 + HRH-1	early embryonic lethality	5.8 ± 2.7
SF2	late embryonic lethality	nd
SRm160 + SF2	late embryonic lethality and increased production of deficient oocytes	37.1 ± 13.5
SRp20	no phenotype	nd
SRm160 + SRp20	increased production of deficient oocytes	43.9 ± 12.1
SRp40	no phenotype	nd
SRm160 + SRp40	increased production of deficient oocytes	33.1 ± 13.6

(*rsr-1*) and CeSF2/ASF (*rsp-3*), ~40% of the scored progeny also contained deficient oocytes, whereas the remaining progeny presented the same late embryonic lethal phenotype observed previously with individual suppression of CeSF2/ASF (Figure 4 and [29]).

The phenotype observed when CeSRm160 and CeSR family proteins were simultaneously suppressed was specific and did not occur as a consequence of ablating the expression of CeSRm160 in combination with other *C. elegans* genes. For example, simultaneous suppression of CeSRm160 and the gene coding for collagen (*col-38*), which gives no phenotype when individually suppressed, resulted in no phenotype and did not lead to the production of deficient oocytes (Figure 4; C. Ibañez and I.L.J., unpublished data). Moreover, simultaneous suppression of CeSRm160 and other splicing factor-related genes also did not result in the deficient-oocyte phenotype. For example, suppression of CeSRm160 and CeHRH-1 (*mog-5*), the *C. elegans* homolog of Prp22/HRH-1, a DEAD-box helicase motif-containing splicing factor, did not alter the embryonic lethal phenotype observed when suppressing CeHRH-1 alone [29, 34], nor did it lead to the production of deficient oocytes (Figures 3c and 4).

The observation that simultaneous suppression of CeSRm160 and individual CeSR family proteins results in a distinct phenotype is indicative of multiple functional interactions between these proteins. Consistent with the multiple physical interactions detected between these proteins in HeLa nuclear extract (Figure 2), a likely possibility is that the genetic interactions reflect conserved RS domain-mediated interactions between SRm160 and SR family proteins in *C. elegans*.

The phenotype caused by simultaneous suppression of CeSRm160 and CeSR family proteins can be rescued by the presence of wild-type sperm

Combined RNAi of CeSRm160 and CeSR family proteins causes a maternal phenocopy, resulting in the production of deficient oocytes. This could be due to the production of aberrant oocytes by the injected mother or, alternatively, to a block in the fertilization of normal oocytes. In order to determine whether the observed phenotype is due to a specific defect in oogenesis, sperm development and/or maintenance, or both processes, we asked whether the deficient-oocyte phenotype could be rescued by mating the injected animals with young male adults (Figure 5). Young hermaphrodite worms were injected with a combination of CeSRm160 (*rsr-1*) and CeSRp40 (*rsp-2*)

Figure 5

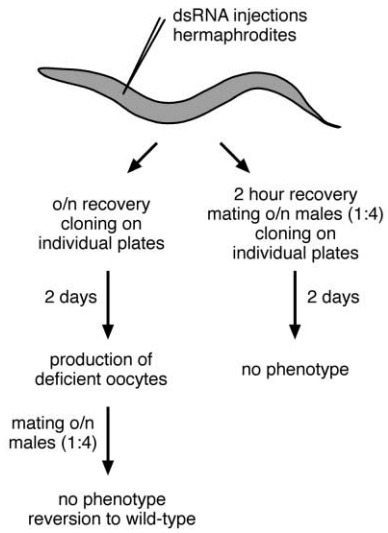


Diagram showing the strategy for the rescue of the dsRNA-induced deficient-oocyte phenotype. Injected hermaphrodites were either mated with young males 3 days after the injection, when the production of deficient oocytes has already occurred (left panel), or were allowed to recover for 2 hr postinjection and subsequently mated overnight with young male adults (right panel).

dsRNA fragments and were mated with young males 3 days after injection, at a time when the production of deficient oocytes has already occurred. Significantly, reversion to wild-type progeny was observed the next day, indicating that the deficient-ooctye phenotype can be rescued by wild-type sperm (Figure 5). In a separate experiment, animals were allowed to recover for 2 hr after injection and were subsequently mated overnight with young male adults. This also resulted in the absence of deficient oocytes and the development of wild-type progeny. Thus, the presence of wild-type sperm (from males) is able to rescue the deficient-oocyte phenotype observed with simultaneous depletion of SRm160 and individual SR proteins. It should be noted that this phenotype is not due to a defect in sperm production, since RNAi injections are performed at a time when sperm production has already been completed.

Thus, this experiment suggests that codepletion of CeSRm160 and CeSR family proteins is affecting one or more steps in the development and/or maintenance of functional sperm and is not a consequence of a specific defect in oogenesis. Unfertilized oocytes have been shown not to complete meiosis and to lack an egg shell. Instead, they undergo multiple rounds of DNA replication and, in the absence of functional microtubule organizing centers, do not undergo mitosis or cytokinesis [35]. Consistent with previous observations that unfertilized oocytes endoreduplicate [35], deficient ooctyes laid by worms in which CeSRm160 and CeSR proteins were targeted by RNAi showed abnormal number of chromosomes by DAPI staining (see Figure S2).

Table 1

RNAi of CeSRm160 in combination with two CeSR genes.

Genes	RNAi phenotype
SRp40 + SC35	no phenotype
SRp40 + SC35 + SRm160	increased production of deficient oocytes
SRp20 + SRp75	vulval defect or vulvaless, sterile, blocked gut, slow growth
SRp20 + SRp75 + SRm160	production of deficient oocytes, embryonic and larval lethality, vulval defect or vulvaless, sterile, blocked gut, slow growth

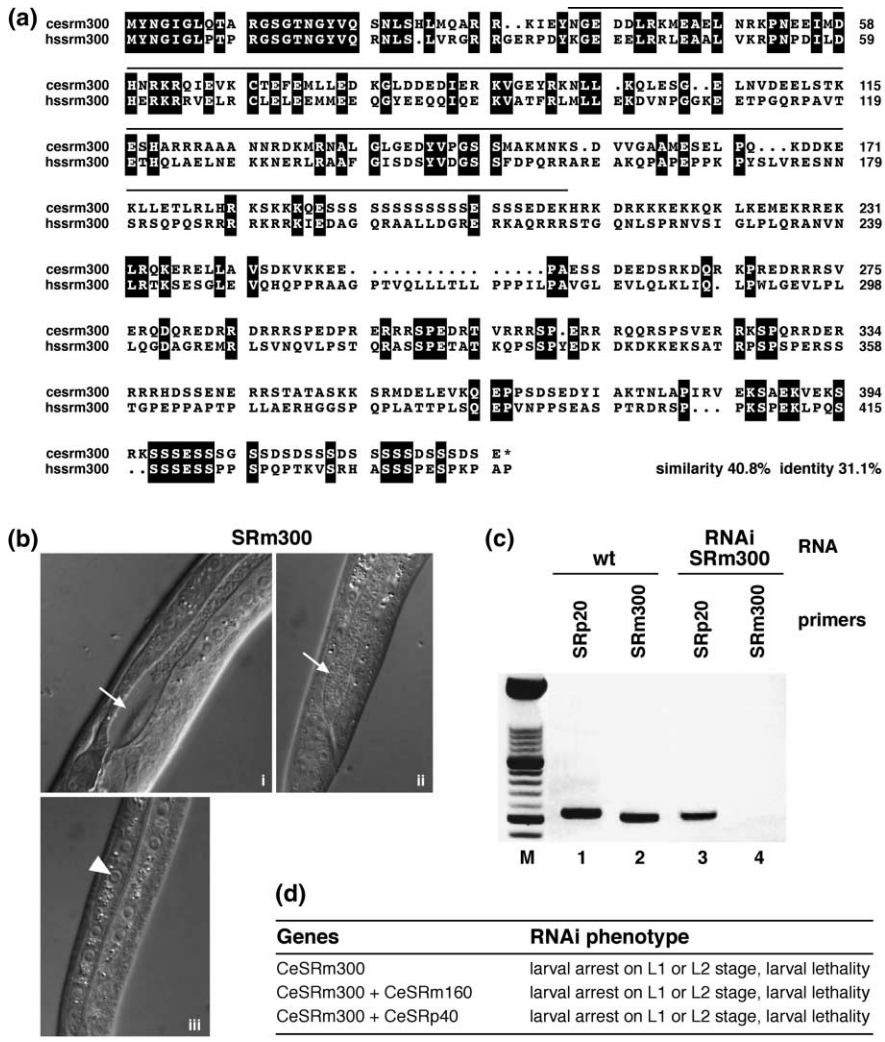
CeSRm160 and SR family genes are also required for postfertilization events

We next performed RNAi of CeSRm160 in combination with two CeSR family genes, whose combined inactivation does or does not lead to a phenotype. As previously observed, RNAi of CeSRp40 (*rsp-2*) and CeSC35 (*rsp-4*) does not give rise to a detectable phenotype (Table 1 and [29]). However, simultaneous suppression of SRm160 with these two CeSR family genes resulted in the production of unfertilized oocytes (Table 1). RNAi of CeSRp20 (*rsp-6*) and CeSRp75 (*rsp-1*) resulted in vulval and gut defects, as well as sterility, as previously reported [29]. Codepletion of CeSRm160, in addition to these phenotypes, also resulted in deficient ooctyes as well as an increase in embryonic and larval lethality (Table 1). Thus, depletion of CeSRm160 together with a combination of CeSR family genes involved in a common pathway (in this case, CeSRp75 and CeSRp20) gives rise to a postfertilization, developmental defect, suggesting that functional interactions between CeSRm160 and combinations of certain CeSR family proteins are important for development.

RNAi of CeSRm300 causes an early larval arrest phenotype that is not affected by simultaneous suppression of CeSRm160

Mammalian SRm160 associates tightly with SRm300, which, like SRm160, lacks a recognizable RRM but contains RS domains as well as numerous other types of repeat motifs that are rich in serine/arginine and/or proline residues [19, 21]. The *C. elegans* genome contains a candidate homolog of human SRm300 (*rsr-2*, identifier Y57A10A.s). The predicted CeSRm300 ORF is highly homologous throughout its length to the human protein (40.8% similarity and 31.1% identity) but is considerably shorter (425 versus 2296 amino acids) and lacks the majority of the repeat sequences found in the human protein including an RS domain (Figure 6a). It was therefore

Figure 6



(d)

Genes	RNAi phenotype
CeSRm300	larval arrest on L1 or L2 stage, larval lethality
CeSRm300 + CeSRm160	larval arrest on L1 or L2 stage, larval lethality
CeSRm300 + CeSRp40	larval arrest on L1 or L2 stage, larval lethality

RNA interference of the CeSRm300 gene. **(a)** Sequence comparison between *C. elegans* (Y57A10A.s) and human SRm300 proteins. Sequences were compared using the GAP program (GCG10 software), and output was produced using PRETTYBOX (GCG10 software). Identical residues are highlighted in black. The dsRNA fragment used for RNAi corresponds to the solid line above the sequence. **(b)** RNA interference with the SRm300 gene leads to early larval arrest and larval lethality. Arrows in panels i and ii indicate a dysfunctional gut full of undigested bacteria. Arrowhead in panel iii indicates an abnormal apoptotic cell nucleus. **(c)** The effectiveness of RNAi was determined by examining the level of the residual transcripts following dsRNA injections by RT-PCR with specific primers, as previously described [29]. CeSRm300 mRNA is specifically depleted in RNAi-treated animals (lane 4) compared to wild-type animals (lane 2), whereas the level of a control mRNA, corresponding to CeSRp20, is unaffected (lanes 1 and 3). The figure shows a negative of an ethidium bromide-stained agarose gel. M, 100 bp ladder DNA size marker. **(d)** Table describing the effects of simultaneous depletion of CeSRm300 with CeSRm160 or CeSR family genes. The CeSRm300 RNAi phenotype was not affected by cosuppression with the CeSRm160 gene or any CeSR family gene.

of interest to investigate whether CeSRm300 interacts genetically with CeSRm160.

RNA interference of CeSRm300 resulted in early larval arrest and larval lethality, possibly due to a dysfunctional gut, which was full of undigested bacteria (Figure 6b, panels i and ii). These affected larvae also display enlarged cell nuclei with a flattened central disc, which are reminiscent of apoptotic cell nuclei (Figure 6b, panel iii). RT-PCR analysis showed that CeSRm300 mRNA was efficiently and specifically depleted in affected F1 animals, indicating that the phenotype was the consequence of inactivation of SRm300 (Figure 6c). This phenotype was not affected by simultaneous suppression of CeSRm160 or individual CeSR family proteins (Figure 6d and data not shown). This indicates that CeSRm300 does not interact genetically with SRm160 in *C. elegans*, which may relate to the observation that it lacks an RS domain and to our previous observation that depletion of human SRm300 does not significantly affect the splicing of different pre-mRNAs in vitro [21]. Moreover, these results further demonstrate that the accumulation of unfertilized oocytes is a specific phenotype resulting from the combined interference of CeSRm160 and CeSR family proteins and not other factors.

Discussion

In this study, we provide evidence that mammalian SRm160 (the SR-related nuclear matrix protein of 160 kDa) interacts with multiple SR family proteins and is required for a large population of different exonic splicing enhancer (ESE) sequences within a random population to promote splicing in vitro. Using RNAi to specifically suppress the activity of the homologs of these proteins in *C. elegans*, we provide evidence that interactions between CeSRm160 and multiple CeSR family proteins are required for fertilization and also for the proper development of the worm.

In previous studies, SELEX-based methods allowed the identification of short and highly degenerate sequences that function as ESEs to promote splicing in conjunction with individual SR proteins. It was also found that a highly diverse set of sequences could function as ESEs, corresponding to as many as 20% of the sequences within a random 18- or 20-mer [24, 36, 37]. In agreement with these results, we have shown that a large population of the sequences within a randomized 18-mer function to promote splicing of the *dsx* pre-mRNA. Depletion of SRm160/300 prevented the majority of splicing activity promoted by sequences in the random population. Previous results demonstrated that SR family proteins are important for the association of SRm160 with pre-mRNA and that different SR family proteins promote splicing in conjunction with different consensus ESE sequences [19, 23, 24]. Taken together with those results, we propose that SRm160 functions as a more general coactivator of ESE-dependent splicing by interacting with many different SR family proteins bound to distinct ESEs. Supporting this model is our finding in the present study that, in the presence of low salt concentrations (0.1 M), SRm160 interacts specifically with several SR family and SR-related proteins.

RNA interference has been widely used as a tool for selectively interfering with gene expression, not only in *C. elegans*, but also in *Drosophila*, plants, and mammalian cells in culture (reviewed in [38]). Introduction of double-stranded RNA results in a drastic reduction in the level of mRNA of the corresponding endogenous gene in a highly sequence-specific manner and has been shown to phenocopy strong loss of function or null alleles of the targeted gene [39]. Thus, RNAi constitutes a powerful reverse genetic tool to probe the function of individual genes.

We have used RNA interference to selectively interfere with SRm160 and SR gene expression in the nematode *C. elegans*, in order to investigate interactions between these splicing regulators in a whole-organism context. We showed previously that individual SR family proteins, with the exception of CeSF2/ASF (*rsp-3*), are functionally redundant in *C. elegans* [29]. One possibility is that the functional redundancy among SR family proteins is related to the high level of degeneracy found in functional ESE sequences, which might allow their recognition by more than one member of the family [24, 29, 33]. We found that simultaneous suppression of CeSRm160 and any one of the CeSR family proteins but not the homologs of other splicing factors resulted in a common phenotype: the production of deficient oocytes by the injected mother. This effect is specific, since it is only obtained with the combination of SRm160 and individual SR proteins, and represents a maternal phenocopy, as opposed to a zygotic effect. This defect was attributed to the absence of functional sperm, since the deficient oocytes could be rescued in mating experiments with wild-type male adults. This raises the possibility that codepletion of SRm160 and individual SR proteins is affecting one or more splicing events that are required for proper sperm development and/or maintenance. It is also possible and perhaps more likely that simultaneous suppression of SRm160 and individual SR proteins could be affecting multiple splicing events, which would cause a decrease in general fitness of the injected worms, which in turn leads to the decreased ability of endogenous sperm to fertilize oocytes. The occurrence of a common phenotype observed with codepletion of CeSRm160 and any individual CeSR protein points to multiple specific interactions between these factors. One possible explanation for the occurrence of this common phenotype is that SRm160 interacts functionally with a multiprotein complex consisting of several SR family proteins. This putative multi-SR family protein complex might compensate for the absence of SRm160, but codepletion of any individual SR protein would lead to its destabilization, thereby resulting in a greater dependency on SRm160. Depletion of SRm160 together with two SR family genes shown to be involved in a common pathway (e.g., SRp75 and SRp20) resulted in drastic developmental defects, in addition to the deficient oocyte phenotype. Therefore, interactions between CeSRm160 and CeSR family proteins are likely to be important for multiple processes at different developmental stages. These experiments showed that, in the absence of CeSRm160, CeSR family proteins are no longer functionally redundant and that codepletion of SR family proteins can have drastic consequences for fertilization and development. These RNAi experiments provide indirect support for the proposed role of SRm160 as a more general coactivator of ESE-dependent splicing.

In addition to *cis*-splicing, *trans*-splicing has been found to occur in a considerable percentage of *C. elegans* transcripts [40], and SR family proteins have been demonstrated to function in this process in the related nematode *Ascaris* [41]. Thus, it is possible that interactions between CeSRm160 and CeSR family proteins are important for both types of splicing. Besides splicing, SRm160 and SR family proteins could have additional functions in mRNA biogenesis. For example, SRm160, together with other proteins including the export factor REF/Aly, has recently been identified as a component of a protein complex that forms upstream of exon-exon junctions dependent on prior splicing [27, 42–44]. In addition, a subset of mammalian SR family proteins shuttle continuously from the nucleus to the cytoplasm, suggesting the involvement of these proteins in one or more postsplicing activities [45]. For example, two of the shuttling SR family proteins, SRp20 and 9G8, promote the export of intronless histone transcripts [46]. Thus, it is possible that SRm160 and/or SR proteins function in one or more downstream processes

that are influenced by prior splicing, such as mRNA export, stability, and/or translation, and that the interactions between SRm160 and SR proteins are also critical for these activities.

In the present study, we also show that RNAi of the *C. elegans* homolog of human SRm300 (the SR-related nuclear matrix protein of 300 kDa), which in mammalian cells interacts tightly with SRm160, leads to early larval arrest and larval lethality. Interestingly, the phenotype observed with RNAi of CeSRm300 was not affected by simultaneous suppression of CeSRm160 or by suppression of any CeSR family protein, arguing against genetic interactions among these proteins. Consistent with our previous biochemical studies demonstrating that efficient depletion of SRm300 does not prevent splicing of different pre-mRNAs [21], it is possible that CeSRm300 contributes a function in *C.elegans* that is not related to the activation of splicing by SRm160 and SR family proteins.

In summary, we have provided evidence that human SRm160 is important for promoting the splicing of a large population of different ESE sequences and that it interacts with several SR family and SR-related proteins. We have also provided evidence that multiple interactions between CeSRm160 and CeSR family proteins are important for proper development in *C. elegans*. Thus, consistent with the high degree of homology between these factors, the results from two very different systems support the conclusion that multiple interactions between SRm160 and SR family proteins provide critical functions, which have been conserved throughout the evolution of metazoans.

Materials and methods

Pre-mRNA templates

Templates for in vitro transcription of the *dsx*ΔE, *dsx*$(GAA)_3$, and *dsx*$(GAA)_6$ substrates are as previously described [22]. For more detailed information, see the Supplementary material.

In vitro transcription and splicing reactions

In vitro transcription was performed in the presence of 6000 Ci/mmole [α-^{32}P]UTP, using SP6 RNA polymerase for the *dsx*(N18) template and T7 RNA polymerase for the *dsx*ΔE, *dsx*$(GAA)_3$, and *dsx*$(GAA)_6$ templates. In vitro splicing assays with the *dsx* substrates were performed as described previously [20]. SRm160/300- and mock-immunodepleted nuclear extracts used in splicing assays were as described and characterized in [19] (see Figure 3 in this previous study). HeLa nuclear extracts, prepared as described previously [47], were purchased from the Computer Cell Culture Centre (C4) (Mons, Belgium).

Immunoprecipitations

Immunoprecipitation of SRm160-containing complexes was performed using a monoclonal antibody specific for SRm160 (B1C8 [26]), exactly as described in [20]. SDS gel electrophoresis and immunoblotting was performed as described previously [26]). The immunoblot was probed with the anti-SR protein antibody mAb104 [25], anti-U1-70K (Cappel), and anti-U2AF-65 [48]. The immunoblot was developed using an anti-mouse secondary antibody conjugated to HRP and chemiluminescence detection (NEN).

dsRNA preparation and microinjection

RNA interference was performed as previously described ([29]) (see Supplementary material)

Rescue of dsRNA-induced phenotype

Young adult hermaphrodites were injected with a combination of SRm160 and SR protein dsRNAs and were split into two groups. The first group was allowed to recover for 2 hr after injection and then were mixed with young males (1:4 ratio). Injected hermaphrodites were cultured with males for 1 day, then cloned onto individual plates, and the phenotype was observed as described above. The second group (control group) was let to recover overnight prior cloning onto individual plates. Alternatively, young males were added onto plates with injected animals after they have developed the expected phenotype, and cultured overnight. The effect on the phenotype was observed as described above.

Supplementary material

Supplementary material containing two additional figures and a more detailed description of Materials and methods is available online at http://images.cellpress.com/supmat/supmatin.htm.

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